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13. ABSTRACT *(Maximum 200*

This project aims to devise strategies to antagonize the promitogenic action of Ras and thereby suppress the transforming activity of the Erb2 oncogene found in 70% of human breast adenocarcinomas. The initial strategy was based on the ability of the Rap-1 GTPase, when overexpressed to suppress the malignant phenotype of V12 Ras-transformed fibroblasts. It was anticipated that Rap-1 which shares an identical sequence corresponding to the primary Ras effector binding domain (amino acids 32-44) when overexpressed, competes with Ras for initial mitogenic effectors. In the past year, we have continued to focus on the identification and characterization of proteins that interact with Rap-1 and Ras through their effector loop in GTP-dependent fashion. We previously described in these reports AF-6 as a polypeptide which binds to Rap-1 in a GTP-dependent fashion, more avidly than to Ras-GTP. We have subsequently identified an additional candidate Ras effector which we have named PRE1. This polypeptide binds Ras-GTP *in vitro* specifically, and more avidly than to Rap-1 GTP. PRE1 is widely expressed, and particularly abundant in the MCF-7 human breast cancer line. The PRE1 isoforms identified thusfar do not contain a catalytic domain, and thus their biochemical function is unknown. Nevertheless, stimulation of human KB cells with EGF promotes a transient association of endogenous PRE1 with endogenous Ras. Consequently, it is likely that PRE1 is a newly discovered Ras effector of significance in epithelial cells.

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Joseph Arnold 9/26/97

PI - Signature Date

Table of Contents

Introduction

Breast cancer is believed to arise from a multistep process involving multiple somatic mutations resulting in the generation of oncogenes and loss of suppressor genes. The process is superimposed on an initial genotype that may contain predisposing mutations (e.g. BRCA1) and is influenced strongly by ovarian steroid hormones (1).

The most common dominant oncogenes found in breast cancer arise from activating mutations in the Erb B2 receptor tyrosine kinase (RTK) resulting in a potent continuous promitogenic signal (1, 2). In all cellular systems examined, the mitogenic capacity of RTKs requires the recruitment of the Ras GTPase (3, 4, 5). Ras itself when mutated to a constitutively active (GTPase deficient) form is a very potent oncogene. Such Ras mutations are very unusual in breast cancer (6), but cRas is frequently overexpressed (2), and this phenotype, when it occurs concomitant with Erb B2 activation, a mutation that occurs in 70% of breast cancers, is associated with a particularly poor prognosis (2).

The overall goal of the studies proposed is to better understand the biochemical mechanism by which RTKs, acting through Ras, promote growth in the breast epithelial cell, and to identify strategies that can be used to antagonize the promitogenic signal conveyed by Ras.

Approximately 4 years ago, work from this and several other laboratories (reviewed in 7) uncovered the first direct evidence as to the biochemical mechanism by which activated Ras promotes cell growth. We showed that the active, GTP bound form of Ras bound directly to regulatory domain of the protein (Ser/Thr) kinase protooncogene, cRaf-1; in fact all three members of the Raf subfamily (ARaf and BRaf) exhibit this interaction. Moreover, introduction of activated, Ras independent forms of Raf into fibroblasts led to transformation. Earlier work from
our lab had shown that a major substrate of the Raf protein kinase is the dual
specificity kinase known as MAPK kinase or MEK (8), which is the immediate activator of the MAP kinase. In fact, several groups showed that expression of constitutively activated forms of MEK was sufficient to give transformation of murine fibroblasts.

These results suggested that the ability of Ras to bind Raf, recruit it to the plasma membrane and initiate its activation provided a sufficient explanation for Ras' potent transforming action, and strategies that interfered with the Ras/Raf interaction were likely to be antimitogenic.

One plausible strategy that provided the initial focus for the present proposal was suggested by the properties of the Ras-related small GTPase, Rap-1. Rap-1 is about 50% identical to Ras in amino acid sequence, and completely identical over the amino acids corresponding to the so-called Ras effector domain, (Ras residues 32-44) (reviewed in 9). Rap-1A was first isolated as a cDNA capable of causing reversion of the morphologic and growth phenotype of V12 Ras transformed fibroblasts (9). The ability of Rap-1 overexpression to revert Ras transformation, together with the identity of the Rap effector domain to that of Ras suggested that Rap-1 might bind Ras' mitogenic effectors, and sequester them in an inactive state. In fact we were able to show that Rap-1 is capable of binding c-Raf in a yeast expression system, in a manner similar to Ras (7). These findings led us to propose that Rap-1, which was known to be expressed in breast, might be recruited to serve as an endogenous Ras antagonist. We therefore proposed to:

1. Create antibody and cDNA reagents necessary for the study of Rap-1 in normal and malignant breast epithelia.
2. Characterize the regulation of the Raf-MAP Kinase pathway in normal and malignant breast epithelia.
3. Examine the effects of Rap-1 overexpression on Ras-directed signal transduction and cell growth.
4. Examine the regulation of Rap-1 activity by extracellular agonists.
5. Determine the nature of the major Rap-1 targets and their relation to Ras action.
6. Examine the control of Rap-1 gene and polypeptide expression in normal and malignant breast tissue.

As described in the 1st years progress report, we had created a satisfactory polyclonal anti Rap-1 anti serum (Task1) and initiated studies on the regulation of Rap-1 *in situ* and its interaction with Raf-1 (Tasks 2, 4). In addition, we had initiated an expression cloning effort to isolate the major Rap-1-associated cellular proteins that might account for Rap-1's anti Ras action, and other of Rap-1's cellular effects. This latter effort (Task5) proved to be unusually productive, and led to the isolation of several sets of novel candidate Rap-1 and Ras interacting proteins. Two of these sets comprised polypeptides whose sequence encoded motifs that gave clear indication of their catalytic function. One set, of course, were the Raf family kinases, whose binding to Rap-1 had been observed by us, and whose role in Ras signaling is well established. A second set of Rap-1 interactors consisted of four distinct polypeptides which each encoded a domain homologous to the catalytic domain of guanyl nucleotide exchange proteins for small GTPases of the Ras superfamily. These four proteins included:

1. cDNAs identical to those previously reported as encoding a Ral specific exchanger (Ral GDS) (10-13).

2. cDNAs sharing about 60% identity to Ral GDS, reported by several groups as Ral GDS-like (RGL) (11).

3. cDNA encoding a protein about 30% identical to Ral GDS and RGL, which was recently reported as RLF (14).

4. One novel, as yet unreported polypeptide which contains a GDS catalytic domain approximately 30% identical to several functionally characterized GDS enzymes, including the Ras specific in m-SOS, the Rap-1 specific C3G, etc.

In addition to these two categories of catalytic polypeptides, we recovered 5 other categories of cDNA encoding polypeptides which lacked catalytic domains, although several encoded protein domains known to be important in protein-protein or protein-lipid interaction, such as a zinc finger domain, a pleckstrin homology domain, ankyrin (ANK) repeats, etc.

Each of these polypeptides, like Raf, interacted both with Rap-1 and Ras in a yeast expression system, in an effector domain-dependent way, indicating that the bound preferentially to the GTP charged forms of the GTPases, and like Raf, were candidate effector molecules. At this point we were faced with the choice of devoting effort primarily to the further characterization of these new candidates (Task5) or proceeding with the descriptive studies of Rap-1 overexpression and regulation (Tasks 2 and 3).

Our decision was strongly influenced by emerging reports from a number of laboratories which indicated that multiple Ras-activated pathways in addition to Raf appeared be required for the transforming action of Ras in many cell backgrounds, (15, 16). Moreover, activated Raf was not capable of transforming a variety of epithelial cell lines that were potently transformed by V12 Ras, including the MCF-10A human breast epithelial cell line (17). This result indicated that although Raf remained an important effector of Ras, other mitogenic effectors, remained to be identified, including elements that were especially important in breast and other epithelial lines (e.g. colonic epithelia, etc.). We therefore elected to focus our effort on the characterization of the novel candidate Ras and Rap-1 effectors we had cloned during the initial period. Last year we reported on the characterization of AF-6, a widely expressed 1612 amino acid protein that binds Rap 1-GTP (in preference to Ras-GTP), in a segment near its aminoterminus. AF-6 has no catalytic function, but contains a PDZ domain, a motif known to bind to the carboxyterminal valine of several polypeptides, as well as two domains homologous to those found in unc104 and myosin type V. This past year has been devoted to the characterization of a new candidate, PRE-1.

Body:

The polypeptide we have named PRE-1 was recovered as a cDNA during two-hybrid screens using both Ras (from a brain library) and Rap-1 (from a T cell library). In the Ras screen, one million yeast transformants yielded twenty Ras interactors, of which eighteen were either c-Raf or A-Raf, and two corresponded to PRE-1.

The 2.5 KB PRE-1 cDNA insert was used as the hybridization probe to isolate the entire cDNA from a mouse brain cDNA library. The open reading frame from the first ATG (methionine) includes 413 amino acids, yielding a highly basic polypeptide (PI=9.41) with a predicted molecular weight of 46.4 KD. One obvious structural feature of Pre-1 is the presence of a cystein-histidine rich segment typical of a diacylglycerol/.phorbol ester (DAG_PE) binding site. PRE-1 also has a proline rich region in its aminoterminal region, with five PXXP sequences, which are possible SH-3 domain binding sites (30).

PRE-1 mRNA abundance and complexity in murine tissues was examined by Northern blot. A single mRNA generally about 3.1 KB was detected in most mouse tissues, although some size variation is noted. The highest levels are observed in brain, liver and spleen, with barely detectable levels in heart.

A polyclonal antibody was raised against a carboxyterminal fragment of PRE-1 and purified by affinity chromatography using the recombinant antigen. Immunoblot of extracts prepared from different rat tissues (figure 1) show a single immunoreactive band at 46 KD in a brain extract, which is in agreement with the predicted size of the polypeptide encoded by PRE-1 cDNA isolated from the mouse brain library. A similar 46 KD band was also seen in other tissues including lung and testes. In addition, however, prominent immunoreactive bands at other molecular weights are seen in most tissues, and some tissues lack a 46 KD band entirely (e.g. skeletal muscle, heart, spleen and liver). All tissues but brain show a major 65 KD band, and two bands around 55 KD are also seen in lung, spleen, testes and liver. The 65 and 55 KD bands may represent isoforms of PRE1, the existence of which is suggested by partial cDNAs we have isolated from a variety of cDNA libraries. Alternatively, these bands may reflect polypeptides unrelated to PRE-1, except for the presence of sequence epitopes recognized by the polyclonal antibodies to PRE-1. The murine brain PRE-1 cDNA was tagged at the PRE-1 aminoterminus with an HA epitope and expressed transiently in COS cells. HA-PRE-1 shows the expected size of 46 KD by immunoblot with anti-PRE1 antibodies (figure 2). Extracts prepared from several cell lines were subjected to PRE-1 immunoblot; of the cell lines examined, only BC3H1, a vascular smooth

muscle-like line derived from a radiation induced murine brain tumor, shows a single band at 46 KD. A band of similar size is seen in several other cell lines including RIE-1 (rat intestinal epithelial), MCF-7 (human breast cancer), HEK 293 (human embryonic kidney) and KB (human oral carcinoma); however, immunoreactive polypeptides of 55 KD (RIE-1, MCF-7, HEK 293 and KB) and 65 KD (RIE-1, HEK 293 and KB), are as or more abundant in these cell lines, and some lines show only bands other than the 46 KD polypeptide (e.g., Huh-7, 40 KD, L6, 55KD), (figure 2).

A GST-PRE-1 fusion protein (corresponding to the PRE-1 polypeptide encoded in the initial cDNA isolate) was expressed and purified from *E. Coli*. Prokaryotic recombinant c-H-Ras was loaded with GTP-γ-S or GDP-β-S and various amounts were mixed with a fixed amount of GST-PRE-1 or GST as control. After incubation at 30^0 C for 20 minutes, GST or GST fusion proteins and any associated proteins were recovered by addition of glutathione-sepharose beads. The beads were washed and eluted into SDS sample buffer; proteins were separated by SDS-PAGE, transferred to PVDF membrane and probed for Ras using a monoclonal anti-Ras antibody. GST-PRE-1, but not GST bound Ras and considerably more Ras-GTP-γ-S is bound than Ras-GDP-β-S (figure 3). These results establish that the effector loop-dependent interaction between PRE-1 and Ras identified by two-hybrid techniques reflects the direct binding of the two proteins, and that the binding between PRE-1 and Ras is GTP-dependent.

We next attempted to detect and *in situ* association between endogenous Ras and endogenous PRE-1, under conditions where the levels of two polypeptides are not increased artificially by transient overexpression. We chose to examine the human oral carcinoma cell line KB because PRE-1 expression is readily detectable and these cells express substantial numbers of EGF receptors. KB cells grown to 80% confluence were serum starved for 24 hours, and then treated with EGF for various times. Triton X-100 soluble cell lysates were subjected to immunoprecipitation using the monoclonal anti-Ras antibodies, Y13-238, which are known to enable isolation of Ras-Raf complexes. The Ras immunoprecipitates were washed extensively with the lysis buffer, eluted into SDS sample buffer and subjected to SDS-PAGE, transfer to PVDF membrane and immunobloted with the affinity purified polyclonal anti-PRE-1 antibodies. Although equal amounts of endogenous Ras was recovered in all samples, the Ras immunoprecipitates contain immunoreactive PRE-1 only after treatment of the cells with EGF (figure 4).

Conclusion:

We have identified PRE-1, a potential new Ras effector or target, using the yeast two-hybrid

screen with Ras as bait. We show that Pre-1 can bind Ras directly *in vitro* using purified recombinant Ras and PRE-1 polypeptides. The Ras/PRE-1 association *in vitro* depends strongly on Ras being in the GTP-bound form. We show that with yeast two-hybrid assay PRE-1 interacts with Ras 12 V, but not two transformation defective effector loop mutants, Ras 12VΔ3438A and Ras 12V38N. This profile of interaction with Ras is identical to that exhibited by known and potential Ras effectors including Raf, PI-3 kinase, Ral GDS, Rin1 and AF-6 (25, 26). Finally, it is clear that a stimulus-dependent association of endogenous Ras and PRE-1 occurs following EGF receptor activation in KB cells. To our knowledge, PRE-1 is the only other candidate mammalian Ras effector, other than Raf, wherein the endogenous polypeptide has been demonstrated to associate with Ras *in vivo* following receptor activation. Taken together, these properties indicate that PRE1 is very likely to be a physiologic Ras effector.

The overall goal of this work remains unchanged, namely to identify strategies to interfere with Ras directed mitogenesis, which can be used to interfere with the promitogenic action of the activated Erb B2 oncogene. The specific tasks however, have evolved from the initial proposal, because of the significant evidence for the crucial participation of Ras effectors other than Raf in the mitogenic action of Ras in breast epithelia. We intend to systematically characterize the biologic response to these newer candidates Ras and Rap-1 effectors, so as to ascertain whether.

1. They are expressed in normal and malignant breast epithelia.
2. The impact of their overexpression, singly and in combination on the growth properties of breast epithelial and fibroblast cell lines, in comparison to Ras and Rap-1.

We plan to complete the cDNA cloning of the remaining, novel Ras and Rap-1 interacting proteins. In addition, we intend to examine in detail the biologic function of PRE-1 as a candidate Ras effector.

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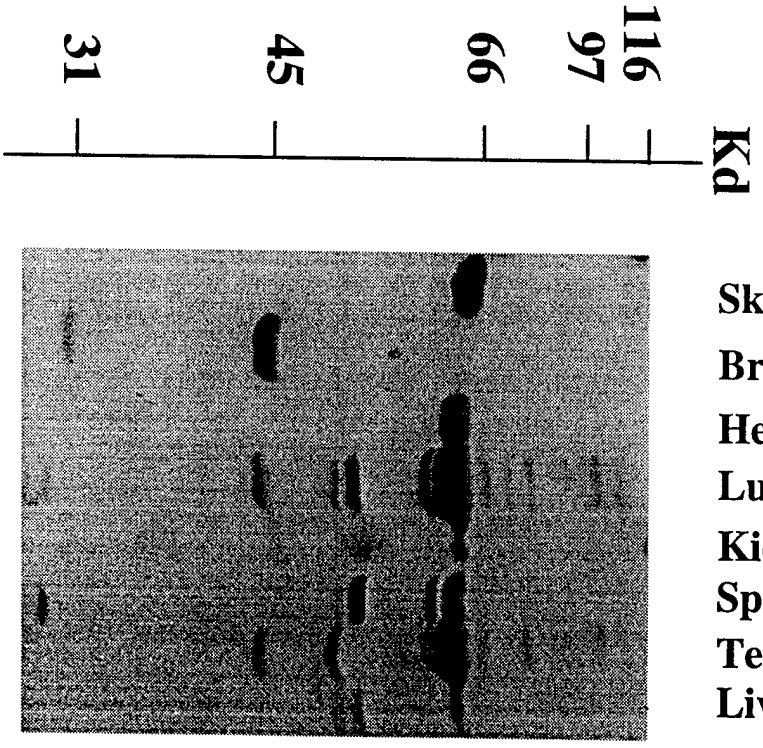
Figure 1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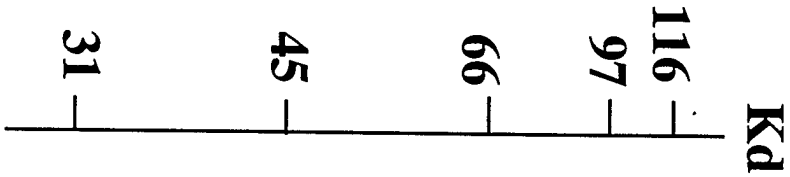

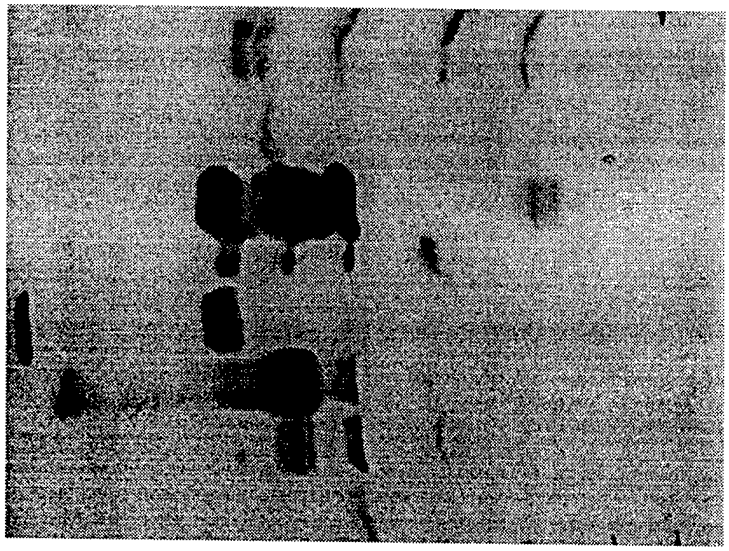

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MCF-7

293

Huh-7

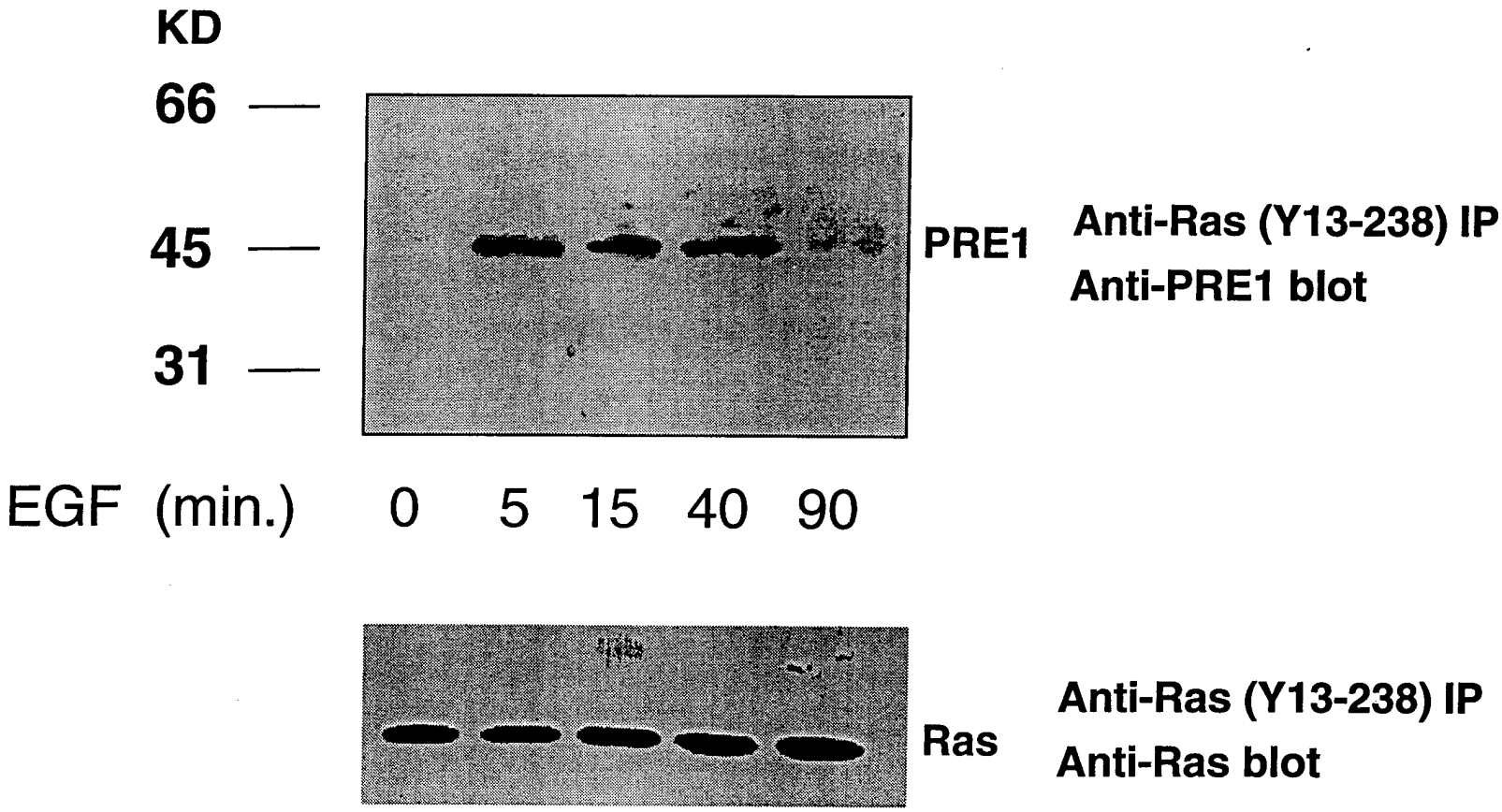
HA-PRE1 in COS

KB

L6

Figure 3

Figure 4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